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Aroclor 1254 and Benzo(a)Pyrene Induced Cyp P450 (A1) in Nile Tilapia Larvae

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ABSTRACT

Background and Objective: Many organisms, including fish, respond to pollutant insult by changing the expression of genes, particularly those for xenobiotic metabolism. The ultimate objective of this study was to provide a practical monitoring tool for assessing exposure to pollutants using Nile tilapia (*Oreochromis niloticus*) as an indicator species. **Materials and Methods:** A total of (60) 6-7 days post fertilized prefeeding larvae of Nile Tilapia (*Oreochromis niloticus*) were used as a model species and exposed to different sublethal concentrations of Aroclor 1254 at a concentration of 16 mg/L and Benzo[a]pyrene, at a concentration of 1.6 mg/L in the laboratory using a quantitative polymerase chain reaction (qPCR) with power SYBR Green II master mixes. Primer sequences of cytochrome P450 A1 gene (CYP 1A forward and reverse) that were already optimized and passed quality criteria were used in the assay. Transcript expressions of genes were measured using RT-qPCR. **Results:** The results showed that each chemical induced significant CYP 1A mRNA gene expression patterns after 24 hrs of exposure, involving biological signaling pathways of aryl hydrocarbon receptor pathways, which are known to respond to chemicals such as PCBs and PAHs compared to control. Each chemical generated a different level of fold change of gene expression in prefeeding larvae of Nile tilapia. **Conclusion:** Thus, this indicates that a standard PCR array such as the one described here, could be used to extend these studies to wild samples (samples from different waterbodies), in order to determine whether contaminants are likely present in harmful concentrations.

KEYWORDS

Aroclor, Benzo[a]pyrene, Nile tilapia, pre-feeding, primers, RT-qPCR, xenobiotics

INTRODUCTION

After more than 40 years of the ban, polychlorinated biphenyl (PCB) remains a contaminant with about 209 congeners identified globally[1]. One good example of such a congener is Aroclor 1254. It is a chemical compound of the class of persistent bioaccumulative toxicants (PTBs), that is referenced by four digits (1254), representing the size of the molecule and the percent mass of the chlorine (12 carbon at the corner of the biphenyl rings) and 54% of the total mass of chlorine from the mixture of congeners[2]. Previous studies have shown that the toxicities of PCBs are mediated through aryl hydrocarbon receptor pathways[2,3]. The PCB 1254 was reported to cause changes in swimming activity, thyroid dysfunction,

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endocrine disruption, liver damage, immunosuppression and carcinogenicity in aquatic organisms[4,5]. Benzo[alpha]pyrene (BaP) is a highly molecular, ubiquitous and hydrophobic organic compound. It comprises of five fused rings in angular, linear and cluster arrangements[6]. It is toxic to cytoplasm, can cause cancer may change genetic makeup and can accumulate in different organs. Due to its cytotoxicity, carcinogenicity, genotoxicity and mutagenicity, it has become a real model of toxicants for studies by various ecotoxicologists[7,8].

Laboratory exposure assessment is the prediction of the duration, frequency and extent to which an organism has been exposed to a certain group or single chemicals in nature. Various forms of exposure are used in laboratory bioassays to determine the toxicity of chemicals in model fish species[9]. Therefore, experimental approaches in the laboratory have an ecotoxicological application, in which the interpretation of the mechanisms of toxic action of xenobiotic metabolism chemicals through the use of gene expression patterns is emphasised[10]. In ecotoxicology, the genomic analysis application in the assessment of xenobiotic metabolism toxicants offer potential by knowing different responses to contaminant exposure involving gene interactions through different metabolic pathways. For example, multiple signalling pathways, receptors and other related genes are involved in the EDC toxicological effect alone in teleost fish.

However, most studies on xenobiotic metabolism chemicals focused on the use of adult fish, leaving behind various developmental stages, such as larval and embryonic stages, which are critical in the interpretation of the xenobiotic metabolism chemical mechanisms of action effects in biomonitoring the aquatic ecosystem[11-13]. One of the most frequently studied CYP isoforms in fish is the Cytochrome P4501A (CYP1A) gene family. Having been split from CYP2 about 450 million years ago, CYP1A has a crucial role in the oxidative biotransformation of various persistent environmental aromatic hydrocarbons, such as planar halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs)[14]. The induced expression of CYP 1A is mediated through the aryl hydrocarbon receptor (AhR) signalling pathway and the initial induction response needs binding to the aryl hydrocarbon receptor.

Bioassays like this (*in vivo*) are frequently used with a reliable endpoint (gene expression)[15]. Since particular genes respond to specific pollutants, it may be possible, by using this type of multiple PCR assays, to determine whether pollutants are present in water bodies at biologically relevant levels. An approach like this has been applied to zebrafish larvae[16,17]; to Western mosquitofish (*Gambusia affinis*)[18]; and to Nile tilapia (*Oreochromis niloticus*)[19].

This approach has allowed for the identification of specific expression profiles caused by particular pollutant treatments linking gene expression profiles and mechanisms of toxicity in fish, as well as getting valuable information on the screening of novel chemical pollutants (potential toxicity) in fishes. It also provides an insight into the future development of systems to assess the comprehensive biochemical responses of fish exposed to mixtures of toxicants/chemicals in a wild aquatic ecosystem and to which classes of chemicals the fish may be exposed, as well as to assume the biomarkers for such exposure or effect[18]. The choice of Nile tilapia larvae in this study is to extend this approach. The post-fertilisation larvae were chosen, because the early life stages of fish are potentially useful as an alternative experimental model as they contain all the important organs as well as one of the vulnerable stages in the lifecycle of teleost[20,21].

The aim of this study was to determine specific gene expression fingerprints for prototypical pollutants using exposing tilapia prefeeding larvae with prototypical environmentally relevant chemical contaminants to a sublethal level in the laboratory to assess the transcriptional responses following exposure to two different chemicals prevalent in Nigerian water bodies (Aroclor 1254 and Benzo(a)pyrene). In this study, the role of CYP 1A in the biotransformation of exogenous chemicals due to exposure to different classes of halogenated aromatic hydrocarbons through CYP1A-AhR signalling pathways was explored.

MATERIALS AND METHODS

Study area: This study was conducted at the Institute of Aquaculture, University of Stirling Tropical Aquarium Scotland, United Kingdom. The study lasted for a period of four months from April- August 2019.

Primer design and optimisation: Gene specific primers (*Cytochrome P4501A* (*CYP 1A*), *Pan Ribosomal protein S5* and *Pan Ribosomal protein S7*) were designed and optimized and were used for the assays to amplify genes of interest in Tilapia.

Chemicals and reagents: All chemicals, kits and reagents used in this study were purchased from sigma-Aldrich Co' Thermoscientific, Primer Design, Alpha Labs (UK) and Eurofins Fenomics, Germany.

Experimental animal: The 60 wild-type tilapia (*Oreochromis niloticus*) prefeeding larvae (6-7 days post fertilization, dpf) with an average length of 6.2±0.1 mm were obtained from the University of Stirling Tropical aquarium. The aquarium staff used standard procedures to hatch the larvae. The larvae were obtained from fish with no history of exposure to contamination and all the larvae were of similar age during the experiment. Until the start of the exposure assays, the larvae were kept in the 3 L plastic recirculated vessels, where they were hatched, for 24 hrs. Before adding chemicals, the larvae were transferred to static 250 mL beakers for an acclimation period of 24 hrs. All the beakers were supplied with continuous aeration to maintain dissolved oxygen from the aquarium pump and air stone, with a light regime of 17 hrs light and 7 hrs dark photoperiod at a temperature of 27±2°C.

Stock solution and preparation of chemicals: A set of 2 different analytical grade chemicals (Sigma Aldrich, grade≥98.0 %) were obtained for the exposure assay. These comprised PCB 126 and Benzo(a)pyrene as exposure treatments, while dimethylsulfuroxide (DMSO) was used as a vehicle control at 0.1%. Stock solutions were prepared in pure DMSO mixed appropriately and stored in the dark until use. The stock solutions of the compounds were prepared each, by weighing the solid chemicals on a weighing balance or the liquid chemicals measured using plastic pipettes, to obtain the exposure concentrations, by dissolving 40 mg Aroclor 1254 and 4 mg Benzo(a)pyrene in 2.5mL of pure DMSO to give a nominal concentration of 16 mg and 1.6 mg, respectively. During the experiment, the stocks were diluted in the water (fish culture) where the larvae had hatched, containing the experimental animals. The chemicals were added to the exposure beakers (to achieve lowest observable effect concentrations (LOEC) as estimated from the literature), by pipetting 250 µL stock solution or DMSO control to each 250 mL beaker. These chemical concentrations were chosen to be lower than that expected to cause any significant malformation or mortality but were known to have effects[5,21-23]. The chemicals were mixed homogeneously with the help of air bubbles.

Experimental set up for the treatment: The 60 larvae were equally divided into two different chemicals (10 larvae per group) during the experiment at 160 hrs post fertilization (6-7 dpf). The larvae were transferred to 250 mL beakers for acclimation for 24 hrs in 3 L fish culture at a temperature of 27±2°C. For each chemical, three groups were set up (two treatment groups and a control), containing ten larvae each. Controls were pure DMSO (vehicle control) diluted in fish culture. From each chemical stock solution prepared earlier, 250 µL was added to different 250 mL beakers by pipetting. Also, 250 µL pure DMSO was pipetted into 250 mL beakers containing fish culture as a vehicle control. After 24 hrs exposure, the larvae were euthanized with an anaesthetic overdose of Benzocaine (3 mL in 1 L of water), collected individually in 2 mL of microtubes containing RNA later and kept frozen at -20°C before RNA extraction. Subsequently, all the water containing the chemicals was collected in a 20 L polypropylene carbon bucket activated charcoal teabags. The 5 days later the water was poured down the drain, retaining the teabags for specialist disposal. The protocol followed was in accordance with the University of Stirling AWERB and UK Home Office guidelines and ensured that all exposed larvae were sacrificed before first feeding.

RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction: Each prefeeding larvae was added to a 2 mL screw cap microtube (Alpha labs) containing 1 mL TriReagent (Sigma, UK) extraction buffer according to the manufacturer's protocols and homogenised using a mini bead beater 24 (Bio spec product) until they were disrupted. The RNA was extracted according to the manufacturer's protocol. The RNA (1µg) was reverse transcribed to produce cDNA, using a Precision Nanoscript 1 reverse transcription kit (Primer design) according to the manufacturer's instructions.

Real-time PCR was performed using a TOptical Thermocoicler® PCR machine (Biometra, Germany). Each 10 µL reaction contained a mastermix reaction of 0.2 µL, 10 µM forward and reverse primers (Eurofins genomics, Germany), 5 µL SYBR Green Luminarissupermix (Thermoscientific), 2.6 µLMiliQ water and 2 µL of the diluted cDNA samples. The following cycling conditions were used: Initial heating at 50°C for 2 min, followed by 95ºC for 10 min for initial incubation to activate the DNA polymerase in the mix. Then, at 95°C for 10 sec for denaturation, at 60°C for 10 sec and 72°C for 15 sec for annealing and extension steps.

Statistical analysis: The statistical analysis of the results was done using the delta-delta Ct method (Pfaffl model equation, 2001) in Excel spreadsheets and SPPSS version 25 (SPSS. Inc. Chicago, USA). Geometric means of the two housekeeping genes (Rps 5 and Rps 7) were used to normalise the target genes difference for each sample. Later the $2^{-\Delta\Delta Ct}$ method was used to determine the relative quantity in real time PCR[24]. All quantitative values were graphed as the mean of the normalised value±standard deviation. The Kolmogorov-Smirnov test was used to check the approximate normal distribution of the data. The resulting data failed normalisation tests even after log transformation and therefore, the non-parametric Kruskal Wallis and *post hoc* tests were used to compare values of gene expression between the treatments and controls of the different chemicals under the same exposure time in different genes. The level of difference was considered significant at $p \leq 0.05$.

RESULTS

Fish survival in chemical exposure: For the determination of the amplification efficiencies of two sets of primers during optimisation, the transcript, accession number, primer name, sequences and G+C content were determined (Table 1). Following the completion of the exposure time (24 hrs), all 60 larvae were recovered without any mortality from all the 6 beakers containing 2 treatments with 10 observable larvae in each beaker (250 mL). The expression of mRNAs of prefeeding tilapia after the treatment with Aroclor 1254 at a concentration of 16 mg/250 mL showed a significantly upregulated expression of CYP1A (13-fold), BaP, a polycyclic aromatic hydrocarbon treatment at a concentration of 1.6 mg/250 mL caused a significant increase in CYP1A (5-fold) compared to control (Table 2).

Table 1: Brief detail of quantitative -PCR primers

Transcript	Accession number	Primer name	Sequences (5'-3')	GC content(%)
Cytochrome P450 1A	NM_001279586.1	Qtn CYP 1Af	CGAGGACAGAAAGCTGGA (20)	55
		Qtn CYP 1Ar	AAGGGGCAAGTTGTTCCGAT (20)	50

Qtn: Quantitative Tilapia, F: Forward primer and r: Reverse primer

Table 2: Chemicals measured and nominal concentrations used as LOEC in laboratory exposure of prefeeding Nile tilapia (*Oreochromis niloticus*)

Chemicals	Measured concentration (mg/L)	Nominal concentration (mg/L)
Aroclor 1254	40	16
Benzo(a)pyrene	4	1.6

Table 3: Genes and chemicals treatments, fold difference compared to control using Kruskal Wallis H test

Chemicals	Blast hit	Fold	Kruskal wallis test P≤0.05
Aroclor 1254	CYP IA	13.275	0.000
Benzo(a)pyrene	CYP IA	5.086	0.000

It is to be notedthat there was thus pseudoreplication in the present assay. Out of the two treatment groups (n = 2, A and B beakers containing 10 larvae per pseudoreplicate), the best ten treated fish mRNA from the two pseudoreplicates (5 larvae from each pseudoreplicate) for all the treatments were chosen and analyzed with the 10 mRNA of each control per treatment. The values of the controls for all the treatments were averaged from the 20 individual controls and their means were taken and used as (n = 10). i.e. 10 individuals for each control (Table 3).

DISCUSSION

Expression of AHR ligands CYP IA was observed in two different treatments in the present study. There were differentially expressed CYP IA in response to Aroclor 1254 at a concentration of 16 mg/L and B[a]P at a concentration of 1.6 mg/L. It was established that the expression of CYP 1A elevates in response to PAHs and PCBs as its agonist. These chemicals activate xenobiotic-metabolizing enzymes by binding to aryl hydrocarbon receptors[25]. Significant expressions of this gene in both Aroclor 1254 (13-fold) and B[a]P (5-fold), compared to control are not surprising in larvae of Nile tilapia. However, A CYP1A mRNA's significant induction in the larvae could be due to the compounds at a concentration most probably consistent with the larvae burden of the contaminant. The development of early-life stage toxicities due to PCBs and PAHs has been documented in fish due to CYP1A induction through mediations of aryl hydrocarbon receptor (AHR) pathway activations[3].

This induction could begin as early as the gastrula stage of development in vertebrates[26]. The observed result is an indication of activation of a pathway involving AHR and this speculates that larval early life stage toxicity of Aroclor 1254 could be mediated by the AHR pathway. Attention was given to early life stage laboratory exposure to chemicals in the present study since the majority of the fish in the natural wild environment particularly larval stages are exposed to toxicities of planar aromatic hydrocarbons with recruitment failure as a possible effect. The majority of the responses in fish due to the toxicities of PCBs and PAHs due to exposure and consequent effects include hatching failure, morphometric changes, conceded development of the eye as well as the short life span of the malnourished larvae. All these responses are likely to reduce the effective recruitment of the larval stage of Nile tilapia to the adult stage in the wild. As a consequence, helps in the failure to restore the impacted population and may lead to the eventual death of the affected population in the wild environment. Various reasons can be justified concerning the pertinence of the present result to the possible effect of Aroclor 1254 induces toxicity to Nile tilapia in the natural wild environment. At first, the treatment of the larvae was through a water-borne exposure route with the employment of DMSO as a solvent vehicle at 6–7days postfertilization. There is a likelihood that the larvae population is exposed to these planar aromatic hydrocarbons in a chemically impacted wild environment through transfer from water. As a consequence, the unfertilized eggs could be carrying high levels of these toxicants in the wild environment before their fertilization. However, exposure in the natural wild environment could begin at an early embryonic developmental stage in the impacted locales just like the use of larvae in the present study. This similarity could ensue in the same upregulation of CYP1A response in both the two life stages and possibly higher biological level toxic response in the embryonic stage in the wild.

Secondly in the present study, the larvae were exposed to an individual Aroclor 1254 and B[a]P congeners, unlike in the wild natural environment, where the larvae could be exposed to the mixture of planar aromatic hydrocarbons and other xenobiotic compounds. Numerous studies have indicated the presence of noncoplanar congeners and other pollutants in the natural aquatic environment and impacted the binding of these agonists to AHR[27]. Based on the known functions of AhR activation in overt toxicity and the present results, it is likely that tilapia is sensitive to induced toxicities of Aroclor 1254 and B[a]P at a concentration of 16 and 1.6 mg/L, respectively.

Nonetheless, the inducibility of CYP 1A has been established to be a biomarker of exposure in environmentally exposed fish in the wild. This observation from short-term exposure would be useful in the differential expression of CYP1A in a field sample to infer long-term exposure responses accumulated over months or years in tilapia. The induction of a CYP1A gene in a Nile tilapia larvae sample in the laboratory exposure could reflect the local pollution load of AHR agonists, including PCBs in the wild. A broad marker of any AHR mediated pathway gene involves uptake, metabolism and excretion of a chemical for a response to be detected and thus suggest an active metabolism of that chemical by the gene. As only one gene expression was quantified in the present study, it could not be determined to what extent the uptake of the chemical compounds was into the larvae during exposure. But what was apparent was that, from the observed result of Aroclor 1254-fold difference against control, it could be deduced that the uptake of Aroclor 1254 could be constant throughout the exposure period. This indicates that the nature of the chlorination and the co-planar arrangement of Aroclor 1254 weaken effective degradation by the induced biotransformation enzymes gene. Therefore, metabolization of Aroclor 1254 is more gradual. Due to the strong lipophilic nature of Aroclor 1254, it accumulates in the larvae all the time restarting the aryl hydrocarbon receptor (AHR) and re-inducing CYP 1A. While B[a]P could be easily metabolized in the liver and commences its metabolism through eliciting of CYP1A gene[28]. Biotransformation of B[a]P may lead to the production of a different compound in the larvae which are regarded as carcinogens as these compounds react with protein and bind to the DNA. Such compounds include electrophilic diol epoxides (BPDE)[29].

In the present study, BPDE or other metabolite production may have occurred in the larvae and necessitate the increased expression of CYP1A. The larvae may have metabolized B[a]P to produce B[a]P 7,8 dihydrodiol which is the precursor of BPDE, which could produce a covalent DNA adduct. Although after B[a]P exposure, no metabolite was determined which is beyond the present study. Therefore, the larvae may have metabolized B[a]P and form an adduct biotransformation product. The development of a PCR-based screening array for monitoring the biological exposure of pollutants in tilapia could provide a powerful tool for the monitoring and management of water quality in the tropics, especially in Nigeria where this approach is limited[30].
This study recommends thus:

- Additional studies may help explain some important molecular pathways regulating this gene-mediated mechanism
- Additional studies and mechanisms that can relate the results of gene expression of fish in an individual contaminant treatment in a laboratory to a response from a wild fish in a complex chemical mixture of the aquatic environment are needed

CONCLUSION

The present study demonstrated that prototypical chemical contaminants could induce changes in gene expression in the larvae of Nile tilapia, a non-model species, like those in other fish species exposed to an environmentally relevant chemical at an acute laboratory exposure. This data enhanced our understanding of the development of PCR arrays on multiple gene modulations. The expression of these genes inferred that environmental chemicals significantly affect the expression of genes that are involved in phase I biotransformation. This data also demonstrates that changes in gene transcription levels could be used as biomarkers of an organism's exposure to environmental contaminants.

SIGNIFICANCE STATEMENT

The ultimate objective was to provide a tool for assessing exposure pollutants using Nile tilapia (*Oreochromis niloticus*) as an indicator species. Most of the research in Nigerian polluted water bodies has centered on the measurements of environmental heavy metals, some organic chemicals and physicochemical parameters using simple methods and common equipments due to limited human

and infrastructure resources. So far, very little work has been done on the use of gene expression biomarkers on fish to detect exposure to pollution in Nigeria. Consequently, this research helps to develop a technique for analysing gene expression variables, which can potentially provide "gene expression profiles" of the classes of chemicals present at biologically meaningful levels.

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